

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/22632 A2

(51) International Patent Classification⁷: **C07H 21/00**

(21) International Application Number: **PCT/EP01/10210**

(22) International Filing Date:
5 September 2001 (05.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 44 948.4 12 September 2000 (12.09.2000) DE
101 20 094.3 25 April 2001 (25.04.2001) DE

(71) Applicant: **DEGUSSA AG** [DE/DE]; Bennigsenplatz 1,
40474 Düsseldorf (DE).

(72) Inventors: **BATHE, Brigitte**; Twieten 1, 33154
Salzkotten (DE). **SCHRÖDER, Indra**; Backscheide
21, 33803 Steinhagen (DE). **FARWICK, Mike**; Gus-
tav-Adolf-Strasse 11, 33615 Bielefeld (DE). **HERMANN,**
Thomas; Zirkonstrasse 8, 33739 Bielefeld (DE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA,
ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

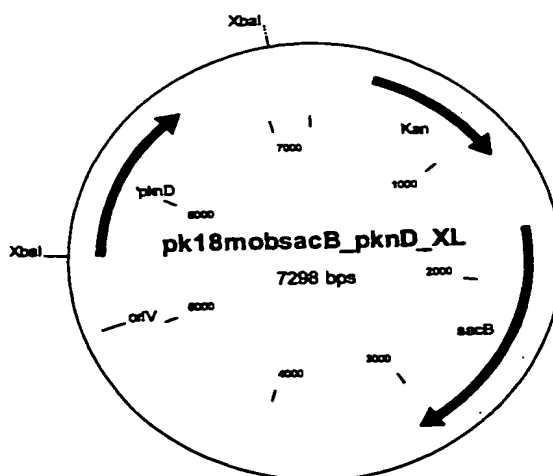
Published:

— without international search report and to be republished
upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: **NUCLEOTIDE SEQUENCES CODING FOR THE PKND GENE**

Map of the plasmid **pk18mobsacB_pknD_XL**



(57) Abstract: The invention relates to an isolated polynucleotide which contains a polynucleotide sequence selected from the group comprising: a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2, b) a polynucleotide coding for a polypeptide containing an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2, c) a polynucleotide which is complementary to the polynucleotides of a) or b), and d) a polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c), and to the use, as hybridization probes, of polynucleotides containing the sequences according to the invention.

Nucleotide Sequences Coding for the *pknD* Gene

Field of the Invention

The invention provides nucleotide sequences from corynebacteria coding for the *pknD* gene and a fermentation
5 process for the preparation of amino acids using bacteria in which the endogenous *pknD* gene is amplified.

Prior Art

L-Amino acids, especially L-lysine, are used in human medicine, in the pharmaceutical industry, in the food
10 industry and very especially in animal nutrition.

It is known that amino acids are prepared by the fermentation of strains of corynebacteria, especially *Corynebacterium glutamicum*. Because of their great importance, attempts are constantly being made to improve
15 the preparative processes. Improvements to the processes may relate to measures involving the fermentation technology, e.g. stirring and oxygen supply, or the composition of the nutrient media, e.g. the sugar concentration during fermentation, or the work-up to the
20 product form, e.g. by ion exchange chromatography, or the intrinsic productivity characteristics of the microorganism itself.

The productivity characteristics of these microorganisms are improved by using methods of mutagenesis, selection and
25 mutant choice to give strains which are resistant to antimetabolites or auxotrophic for metabolites important in regulation, and produce amino acids.

Methods of recombinant DNA technology have also been used for some years to improve L-amino acid-producing strains of
30 *Corynebacterium* by amplifying individual amino acid biosynthesis genes and studying the effect on amino acid production.

Object of the Invention

The object which the inventors set themselves was to provide novel measures for improving the preparation of amino acids by fermentation.

5 Summary of the Invention

When L-amino acids or amino acids are mentioned hereafter, they are understood as meaning one or more amino acids, including their salts, selected from the group comprising
10 L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine is particularly preferred.

When L-lysine or lysine is mentioned hereafter, it is
15 understood as meaning not only the bases but also the salts, e.g. lysine monohydrochloride or lysine sulfate.

The invention provides an isolated polynucleotide from corynebacteria which contains a polynucleotide sequence coding for the pknD gene and is selected from the group
20 comprising:

- a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2,
- b) a polynucleotide coding for a polypeptide containing an
25 amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2,
- c) a polynucleotide which is complementary to the polynucleotides of a) or b), and
- d) a polynucleotide containing at least 15 consecutive
30 nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of protein kinase D.

The invention also provides the above-mentioned polynucleotide, which is preferably a replicable DNA
5 containing:

- (i) the nucleotide sequence shown in SEQ ID No. 1,
or
- (ii) at least one sequence corresponding to sequence
10 (i) within the degeneracy of the genetic code,
or
- (iii) at least one sequence which hybridizes with the
sequence complementary to sequence (i) or (ii),
and optionally
- (iv) neutral sense mutations in (i).

15 The invention also provides:

a replicable polynucleotide, especially DNA, containing the
nucleotide sequence as shown in SEQ ID No. 1,

a polynucleotide coding for a polypeptide containing the
amino acid sequence as shown in SEQ ID No. 2,

20 a vector containing the polynucleotide according to the
invention, especially a shuttle vector or plasmid
vector, and

corynebacteria which contain the vector or in which the
endogenous pknD gene is amplified.

25 The invention also provides polynucleotides consisting
substantially of a polynucleotide sequence which are
obtainable by screening, by means of hybridization, of an
appropriate gene library of a Corynebacterium, containing
the complete gene or parts thereof, with a probe containing

the sequence of the polynucleotide of the invention according to SEQ ID No. 1 or a fragment thereof, and by isolation of said polynucleotide sequence.

Detailed Description of the Invention

- 5 As hybridization probes for RNA, cDNA and DNA, polynucleotides containing the sequences according to the invention are suitable for isolating the full length of nucleic acids, or polynucleotides or genes, coding for protein kinase D, or for isolating nucleic acids, or
- 10 polynucleotides or genes, whose sequence exhibits a high degree of similarity to the sequence of the pknD gene. They are also suitable for incorporation into so-called arrays, micro-arrays or DNA chips for detecting and determining the corresponding polynucleotides.
- 15 Polynucleotides containing the sequences according to the invention are further suitable as primers for the preparation, by the polymerase chain reaction (PCR), of DNA of genes coding for protein kinase D.

- Such oligonucleotides serving as probes or primers contain
- 20 at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24 and very particularly preferably at least 15, 16, 17, 18 or 19 consecutive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45,
- 25 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides may also be suitable.

"Isolated" means separated from its natural environment.

- "Polynucleotide" refers in general to polyribonucleotides
- 30 and polydeoxyribonucleotides, it being possible for the RNAs or DNAs in question to be unmodified or modified.

the sequence of the polynucleotide of the invention according to SEQ ID No. 1 or a fragment thereof, and by isolation of said polynucleotide sequence.

Detailed Description of the Invention

- 5 As hybridization probes for RNA, cDNA and DNA, polynucleotides containing the sequences according to the invention are suitable for isolating the full length of nucleic acids, or polynucleotides or genes, coding for protein kinase D, or for isolating nucleic acids, or
- 10 polynucleotides or genes, whose sequence exhibits a high degree of similarity to the sequence of the pknD gene. They are also suitable for incorporation into so-called arrays, micro-arrays or DNA chips for detecting and determining the corresponding polynucleotides.
- 15 Polynucleotides containing the sequences according to the invention are further suitable as primers for the preparation, by the polymerase chain reaction (PCR), of DNA of genes coding for protein kinase D.

- Such oligonucleotides serving as probes or primers contain
- 20 at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24 and very particularly preferably at least 15, 16, 17, 18 or 19 consecutive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45,
- 25 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides may also be suitable.

"Isolated" means separated from its natural environment.

- 30 "Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, it being possible for the RNAs or DNAs in question to be unmodified or modified.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom, as well as polynucleotides which are in particular at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

10 "Polypeptides" are understood as meaning peptides or proteins containing two or more amino acids bonded via peptide links.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, especially those with the biological activity of protein kinase D and also 15 those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2, and have said activity.

20 The invention further relates to a fermentation process for the preparation of amino acids selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L- 25 arginine, using corynebacteria which, in particular, already produce amino acids and in which the nucleotide sequences coding for the pknD gene are amplified and, in particular, overexpressed.

30 In this context the term "amplification" describes the increase in the intracellular activity, in a microorganism, of one or more enzymes which are coded for by the appropriate DNA, for example by increasing the copy number of the gene(s) or allele(s), using a strong promoter or

using a gene or allele coding for an appropriate enzyme with a high activity, and optionally combining these measures.

5 By amplification measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or
10 the activity or concentration of the protein in the starting microorganism.

The microorganisms provided by the present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch or cellulose or from glycerol and ethanol. Said microorganisms can be
15 representatives of corynebacteria, especially of the genus *Corynebacterium*. The species *Corynebacterium glutamicum* may be mentioned in particular in the genus *Corynebacterium*, being known to those skilled in the art for its ability to produce L-amino acids.

20 The following known wild-type strains:

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
25 *Corynebacterium melassecola* ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869, and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared
30 therefrom, are particularly suitable strains of the genus *Corynebacterium*, especially of the species *Corynebacterium glutamicum* (*C. glutamicum*).

The novel *pknD* gene of *C. glutamicum* coding for the enzyme protein kinase D (EC 2.7.1.37) has been isolated.

The first step in isolating the *pknD* gene or other genes of *C. glutamicum* is to construct a gene library of this
5 microorganism in *Escherichia coli* (*E. coli*). The construction of gene libraries is documented in generally well-known textbooks and manuals. Examples which may be mentioned are the textbook by Winnacker entitled *From Genes to Clones, Introduction to Gene Technology* (Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook et al.
10 entitled *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989). A very well-known gene library is that of the *E. coli* K-12 strain W3110, which was constructed by Kohara et al. (*Cell* 50, 495-508
15 (1987)) in λ vectors. Bathe et al. (*Molecular and General Genetics* 252, 255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was constructed using cosmid vector SuperCos I (Wahl et al., 1987, *Proceedings of the National Academy of Sciences USA* 84, 2160-2164) in the *E.*
20 *coli* K-12 strain NM554 (Raleigh et al., 1988, *Nucleic Acids Research* 16, 1563-1575).

Börmann et al. (*Molecular Microbiology* 6(3), 317-326 (1992)) in turn describe a gene library of *C. glutamicum* ATCC13032 using cosmid pHG79 (Hohn and Collins, *Gene* 11,
25 291-298 (1980)).

A gene library of *C. glutamicum* in *E. coli* can also be constructed using plasmids like pBR322 (Bolivar, *Life Sciences* 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, *Gene* 19, 259-268). Restriction- and recombination-
30 defective *E. coli* strains are particularly suitable as hosts, an example being the strain DH5 α mc^r, which has been described by Grant et al. (*Proceedings of the National Academy of Sciences USA* 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can then in turn
35 be subcloned into common vectors suitable for sequencing,

and subsequently sequenced, e.g. as described by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America 74, 5463-5467, 1977).

The DNA sequences obtained can then be examined with known algorithms or sequence analysis programs, e.g. that of Staden (Nucleic Acids Research 14, 217-232 (1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

10 The novel DNA sequence of *C. glutamicum* coding for the *pknD* gene was found and, as SEQ ID No. 1, forms part of the present invention. Furthermore, the amino acid sequence of the corresponding protein was derived from said DNA
15 amino acid sequence of the *pknD* gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 due to the degeneracy of the genetic code also form part of the invention. Likewise, DNA sequences which hybridize with
20 SEQ ID No. 1 or portions of SEQ ID No. 1 form part of the invention. Furthermore, conservative amino acid exchanges, e.g. the exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are known to those skilled in the art as "sense mutations", which do not cause
25 a fundamental change in the activity of the protein, i.e. they are neutral. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair its function or may even stabilize it. Those skilled in the art will find information on this subject in Ben-Bassat
30 et al. (Journal of Bacteriology 169, 751-757 (1987)), O'Regan et al. (Gene 77, 237-251 (1989)), Sahin-Toth et al. (Protein Sciences 3, 240-247 (1994)) and Hochuli et al. (Bio/Technology 6, 1321-1325 (1988)), inter alia, and in well-known textbooks on genetics and molecular biology.

Amino acid sequences which correspondingly result from SEQ ID No. 2 also form part of the invention.

Likewise, DNA sequences which hybridize with SEQ ID No. 1 or portions of SEQ ID No. 1 form part of the invention.

- 5 Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers resulting from SEQ ID No. 1 form part of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

- 10 Those skilled in the art will find instructions on the identification of DNA sequences by means of hybridization in inter alia the manual entitled "The DIG System User's Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 15 41, 255-260), inter alia. Hybridization takes place under stringent conditions; in other words, only hybrids for which the probe and the target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of 20 hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under relatively low stringency compared with the washing steps (Hybaid 25 Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

- The hybridization reaction can be carried out for example using a 5x SSC buffer at a temperature of approx. 50°C - 68°C, it also being possible for probes to hybridize with polynucleotides which are less than 70% identical to the 30 sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved for example by lowering the salt concentration to 2x SSC and subsequently to 0.5x SSC if necessary (The DIG System User's Guide for Filter 35 Hybridization, Boehringer Mannheim, Mannheim, Germany,

1995), the temperature being adjusted to approx. 50°C - 68°C. It is possible to lower the salt concentration to 0.1x SSC if necessary. By raising the hybridization temperature in approx. 1 - 2°C steps from 50°C to 68°C, it is possible to isolate polynucleotide fragments which are e.g. at least 70%, at least 80% or at least 90% to 95% identical to the sequence of the probe used. Further instructions on hybridization are commercially available in the form of kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1603558).

Those skilled in the art will find instructions on the amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) in the manual by Gait entitled Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994), inter alia.

It has been found that, after overexpression of the *pknD* gene, the production of amino acids by corynebacteria is improved.

Overexpression can be achieved by increasing the copy number of the appropriate genes or mutating the promoter and regulatory region or the ribosome binding site located upstream from the structural gene. Expression cassettes incorporated upstream from the structural gene work in the same way. Inducible promoters additionally make it possible to increase the expression in the course of the production of amino acid by fermentation. Measures for prolonging the life of the mRNA also improve the expression. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs can either be located in plasmids of variable copy number or integrated and amplified in the chromosome. Alternatively, it is also possible to achieve overexpression of the genes in question

by changing the composition of the media and the culture technique.

Those skilled in the art will find relevant instructions in Martin et al. (Bio/Technology 5, 137-146 (1987)), Guerrero
5 et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), Eikmanns et al. (Gene 102, 93-98 (1991)), EP 0 472 869, US 4,601,893, Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132
10 (1994)), LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), WO 96/15246, Malumbres et al. (Gene 134, 15-24 (1993)), JP-A-10-229891, Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) and Makrides (Microbiological Reviews 60, 512-538 (1996)),
15 inter alia, and in well-known textbooks on genetics and molecular biology.

For amplification, the pknD gene according to the invention has been overexpressed for example with the aid of episomal plasmids. Suitable plasmids are those which are replicated
20 in corynebacteria. Numerous known plasmid vectors, e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64, 549-554), pEKEx1 (Eikmanns et al., Gene 102, 93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107, 69-74 (1991)), are based on cryptic plasmids pHM1519, pBL1 or
25 pGA1. Other plasmid vectors, e.g. those based on pCG4 (US-A-4,489,160), pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)) or pAG1 (US-A-5,158,891), can be used in the same way.

Other suitable plasmid vectors are those which make it
30 possible to use the gene amplification process by integration into the chromosome, as described for example by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for the duplication or amplification of the hom-thrB operon. In this method the
35 complete gene is cloned into a plasmid vector which can

replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Examples of suitable vectors are pSUP301 (Simon et al., *Bio/Technology* 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., *Gene* 145, 69-73 (1994)), pGEM-T
5 (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994), *Journal of Biological Chemistry* 269, 32678-84; US-A-5,487,993), pCR[®]Blunt (Invitrogen, Groningen, The Netherlands; Bernard et al., *Journal of Molecular Biology* 234, 534-541 (1993)), pEM1 (Schrumpf et al., 1991, *Journal*
10 *of Bacteriology* 173, 4510-4516) or pBGS8 (Spratt et al., 1986, *Gene* 41, 337-342). The plasmid vector containing the gene to be amplified is then transferred to the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described for example in
15 Schäfer et al. (*Applied and Environmental Microbiology* 60, 756-759 (1994)). Methods of transformation are described for example in Thierbach et al. (*Applied Microbiology and Biotechnology* 29, 356-362 (1988)), Dunican and Shivnan (*Bio/Technology* 7, 1067-1070 (1989)) and Tauch et al. (*FEMS*
20 *Microbiological Letters* 123, 343-347 (1994)). After homologous recombination by means of a crossover event, the resulting strain contains at least two copies of the gene in question.

It has also been found that amino acid exchanges in the
25 section between position 661 and position 669 of the amino acid sequence of protein kinase D, shown in SEQ ID No. 2, improve the production of amino acids, especially lysine, by corynebacteria.

Preferably, L-glutamic acid in position 664 is exchanged
30 for any other proteogenic amino acid except L-glutamic acid, and/or glycine in position 666 is exchanged for any other proteogenic amino acid except glycine.

The exchange in position 664 is preferably for L-lysine or L-arginine, especially L-lysine, and the exchange in

position 666 is preferably for L-serine or L-threonine, especially L-serine.

SEQ ID No. 3 shows the base sequence of the pknD-1547 allele contained in the strain DM1547. The pknD-1547 allele codes for a protein whose amino acid sequence is shown in SEQ ID No. 4. The protein contains L-lysine in position 664 and L-serine in position 666. The DNA sequence of the pknD-1547 allele (SEQ ID No. 3) contains the base adenine in place of the base guanine contained in the pknD wild-type gene (SEQ ID No. 1) in position 2501, and the base adenine in place of the base guanine in position 2507.

Mutagenesis can be carried out by conventional methods using mutagenic substances such as N-methyl-N'-nitro-N-nitrosoguanidine or ultraviolet light. Mutagenesis can also be carried out using in vitro methods such as treatment with hydroxylamine (Miller, J.H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) or mutagenic oligonucleotides (T.A. Brown: Gentechnologie für Einsteiger (Gene Technology for Beginners), Spektrum Akademischer Verlag, Heidelberg, 1993), or the polymerase chain reaction (PCR) as described in the manual by Newton and Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994).

The corresponding alleles or mutations are sequenced and introduced into the chromosome by the method of gene replacement, for example as described in Peters-Wendisch et al. (Microbiology 144, 915-927 (1998)) for the *pyc* gene of *C. glutamicum*, in Schäfer et al. (Gene 145, 69-73 (1994)) for the *hom-thrB* gene region of *C. glutamicum* or in Schäfer et al. (Journal of Bacteriology 176, 7309-7319 (1994)) for the *cgl* gene region of *C. glutamicum*. The corresponding alleles or the associated proteins can optionally be amplified in turn.

In addition it can be advantageous for the production of L-amino acids to amplify and, in particular, overexpress not only the *pknD* gene but also one or more enzymes of the particular biosynthetic pathway, the glycolysis, the anaplerosis, the citric acid cycle, the pentose phosphate cycle or the amino acid export, and optionally regulatory proteins.

Thus, for the production of L-amino acids, one or more endogenous genes selected from the following group can be amplified and, in particular, overexpressed in addition to amplification of the *pknD* gene:

- the *dapA* gene coding for dihydrodipicolinate synthase (EP-B-0 197 335),
- the *gap* gene coding for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086),
- the *tpi* gene coding for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086),
- the *pgk* gene coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086),
- the *zwf* gene coding for glucose-6-phosphate dehydrogenase (JP-A-09224661),
- the *pyc* gene coding for pyruvate carboxylase (DE-A-198 31 609),
- the *lysC* gene coding for a feedback-resistant aspartate kinase (Accession no. P26512; EP-B-0387527; EP-A-0699759),
- the *lye* gene coding for lysine export (DE-A-195 48 222),

- The hom gene coding for homoserine dehydrogenase (EP-A-0131171),
the ilvA gene coding for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or
5 the ilvA(Fbr) allele coding for a feedback-resistant threonine dehydratase (Möckel et al., (1994), Molecular Microbiology 13, 833-842),
the ilvBN gene coding for acetohydroxy acid synthase (EP-B-0356739),
- 10 • the ilvD gene coding for dihydroxy acid dehydratase (Sahm and Eggeling (1999), Applied and Environmental Microbiology 65, 1973-1979),
• the zwal gene coding for the Zwal protein (DE 199 59 328.0, DSM13115).
- 15 In addition to amplification of the pknD gene, it can also be advantageous for the production of L-amino acids to attenuate one or more genes selected from the following group:
 - the pck gene coding for phosphoenol pyruvate
20 carboxykinase (DE 199 50 409.1, DSM13047),
 - the pgi gene coding for glucose-6-phosphate isomerase (US 09/396,478, DSM12969),
 - the poxB gene coding for pyruvate oxidase (DE 199 51 975.7, DSM13114),
- 25 • the zwa2 gene coding for the Zwa2 protein (DE 199 59 327.2, DSM13113),

and, in particular, to reduce the expression.

In this context the term "attenuation" describes the reduction or switching-off of the intracellular activity,

in a microorganism, of one or more enzymes (proteins) which are coded for by the appropriate DNA, for example by using a weak promoter or using a gene or allele coding for an appropriate enzyme with a low activity, or inactivating the appropriate gene or enzyme (protein), and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

It can also be advantageous for the production of amino acids not only to overexpress the *pknD* gene but also to switch off unwanted secondary reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention are also provided by the invention and can be cultivated for the production of amino acids continuously or discontinuously by the batch process, the fed batch process or the repeated fed batch process. A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Bioprocess Technology 1. Introduction to Bioengineering) (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Bioreactors and Peripheral Equipment) (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must appropriately meet the demands of the particular strains. Descriptions of culture media for various microorganisms can be found in "Manual of

Methods for General Bacteriology" of the American Society for Bacteriology (Washington DC, USA, 1981).

Carbon sources which can be used are sugars and carbohydrates, e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, e.g. soybean oil, sunflower oil, groundnut oil and coconut fat, fatty acids, e.g. palmitic acid, stearic acid and linoleic acid, alcohols, e.g. glycerol and ethanol, and organic acids, e.g. acetic acid. These substances can be used individually or as a mixture.

Nitrogen sources which can be used are organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used individually or as a mixture.

Phosphorus sources which can be used are phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium salts. The culture medium must also contain metal salts, e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins can be used in addition to the substances mentioned above. Suitable precursors can also be added to the culture medium. Said feed materials can be added to the culture all at once or fed in appropriately during cultivation.

The pH of the culture is controlled by the appropriate use of basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled using antifoams such as fatty acid polyglycol esters. The stability of plasmids can be maintained by

- adding suitable selectively acting substances, e.g. antibiotics, to the medium. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gaseous mixtures, e.g. air, into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until the formation of the desired product has reached a maximum. This objective is normally achieved within 10 hours to 160 hours.
- 10 Methods of determining L-amino acids are known from the state of the art. They can be analyzed for example by ion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30 (1958) 1190), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51, 1167-1174).

20 A pure culture of the *Corynebacterium glutamicum* strain DM1547 was deposited as DSM 13994 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures (DSMZ), Brunswick, Germany) on 16 January 2001 under the terms of the Budapest Treaty.

25 A pure culture of the *Escherichia coli* strain S17-1/pK18mobsacB_pknD_XL was deposited as DSM 14410 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) on 18 July 2001 under the terms of the Budapest Treaty.

30 The fermentation process according to the invention is used for the preparation of amino acids.

The present invention is illustrated in greater detail below by means of Examples.

The isolation of plasmid DNA from *Escherichia coli* and all the techniques of restriction, Klenow treatment and alkaline phosphatase treatment were carried out according to Sambrook et al. (Molecular Cloning. A Laboratory Manual 5 (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA). Methods of transforming *Escherichia coli* are also described in this manual.

The composition of common nutrient media, such as LB or TY medium, can also be found in the manual by Sambrook et al.

10 Example 1

Preparation of a genomic cosmid gene library from *Corynebacterium glutamicum* ATCC13032

Chromosomal DNA from *Corynebacterium glutamicum* ATCC13032 was isolated as described by Tauch et al. (1995, Plasmid 15 33, 168-179) and partially cleaved with the restriction enzyme *Sau3AI* (Amersham Pharmacia, Freiburg, Germany, product description *Sau3AI*, code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, 20 product description SAP, code no. 1758250). The DNA of cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences USA 84, 2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, code no. 251301), 25 was cleaved with the restriction enzyme *XbaI* (Amersham Pharmacia, Freiburg, Germany, product description *XbaI*, code no. 27-0948-02) and also dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme 30 *BamHI* (Amersham Pharmacia, Freiburg, Germany, product description *BamHI*, code no. 27-0868-04). The cosmid DNA treated in this way was mixed with the treated ATCC13032 DNA and the mixture was treated with T4 DNA ligase

(Amersham Pharmacia, Freiburg, Germany, product description T4 DNA ligase, code no. 27-0870-04). The ligation mixture was then packaged into phages using Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, product description
5 Gigapack II XL Packing Extract, code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al., 1988, Nucleic Acid Research 16, 1563-1575), the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. Infection and titering of the cosmid
10 library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated on LB agar (Lennox, 1955, Virology 1, 190) containing 100 mg/l of ampicillin. After
15 incubation overnight at 37°C, recombinant single clones were selected.

Example 2

Isolation and sequencing of the pknD gene

The cosmid DNA of a single colony was isolated with the Qiaprep Spin Miniprep Kit (product no. 27106, Qiagen,
20 Hilden, Germany) in accordance with the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, product no. 27-0913-02). The
25 DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, product no. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range from 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (product no. 20021, Qiagen,
30 Hilden, Germany).

The DNA of sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, product description Zero Background Cloning Kit, product no. K2500-01), was

- cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, product no. 27-0868-04). Ligation of the cosmid fragments into sequencing vector pZero-1 was carried out as described
- 5 by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then introduced into the E. coli strain DH5 α MCR (Grant, 1990,
- 10 Proceedings of the National Academy of Sciences USA 87, 4645-4649) by electroporation (Tauch et al. 1994, FEMS Microbiol. Letters 123, 343-7) and plated on LB agar (Lennox, 1955, Virology 1, 190) containing 50 mg/l of zeocin.
- 15 Plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (product no. 900200, Qiagen, Hilden, Germany). Sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences USA 74, 5463-5467) with
- 20 modifications by Zimmermann et al. (1990, Nucleic Acids Research 18, 1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (product no. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the sequencing reaction
- 25 were carried out in a "Rotiphorese NF acrylamide/bisacrylamide" gel (29:1) (product no. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).
- 30 The raw sequence data obtained were then processed using the Staden programming package (1986, Nucleic Acids Research 14, 217-231), version 97-0. The individual sequences of the pZero-1 derivatives were assembled into a cohesive contig. Computer-assisted coding region analysis

was performed with the XNIP program (Staden, 1986, Nucleic Acids Research 14, 217-231).

The nucleotide sequence obtained is shown in SEQ ID No. 1. Analysis of the nucleotide sequence gave an open reading
5 frame of 2223 base pairs, which was called the pknD gene. The pknD gene codes for a protein of 740 amino acids.

Example 3

Preparation of a replacement vector for replacement of the pknD alleles

10 Chromosomal DNA was isolated from the strain DSM13994 by the method of Eikmanns et al. (Microbiology 140:1817-1828 (1994)). On the basis of the sequence of the pknD gene known for *C. glutamicum* from example 2, the following
15 oligonucleotides were chosen for the polymerase chain reaction (see also SEQ ID No. 5 and SEQ ID No. 6):

pknD_XL-A1:

5' (tct aga) cgg ttg gtg gtt cgg ttc ag 3'

pknD_XL-E1:

5' (tct aga) agc ggc aat gcc ggt gag ta 3'

20 The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the PCR method of Karreman (BioTechniques 24:736-742, 1998) with Pwo-Polymerase from Boehringer. The primers pknD_XL-A1 and pknD_XL-E1 each contain an inserted cleavage
25 site for the restriction enzyme XbaI, these being indicated in parentheses in the representation. With the aid of the polymerase chain reaction, a 1.6 kb DNA section is amplified and isolated, this carrying the pknD gene or allele.

30 The amplified DNA fragment of approx. 1.6 kb length, which carries the pknD allele of the strain DSM13994, was cleaved with the restriction enzyme XbaI, identified by

electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by the conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The plasmid pK18mobsacB (Jäger et al., Journal of
5 Bacteriology, 1:784-791 (1992)) was also cleaved with the
restriction enzyme XbaI. The plasmid pK18mobsacB and the
PCR fragment were ligated. The E. coli strain S17-1 (Simon
et al., 1993, Bio/Technology 1:784-791) was then
10 electroporated with the ligation batch (Hanahan, In: DNA
Cloning. A Practical Approach. Vol. I, IRL-Press, Oxford,
Washington DC, USA, 1985). Selection of plasmid-carrying
cells was carried out by plating out the transformation
batch on LB Agar (Sambrook et al., Molecular cloning: a
15 laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, N.Y., 1989), which had been
supplemented with 25 mg/l kanamycin. Plasmid DNA was
isolated from a transformant with the aid of the QIAprep
Spin Miniprep Kit from Qiagen and checked by restriction
20 with the restriction enzyme XbaI and subsequent agarose gel
electrophoresis (0.8%). The plasmid was called
pK18mobsacB_pknD_XL and is shown in Figure 1.

Brief Description of the Figure:

Figure 1: Map of the plasmid pK18mobsacB_pknD_XL.

25 The abbreviations and designations used have the following
meaning. The length data are to be understood as approx.
values.

sacB:	sacB gene
oriV:	Replication origin V
KmR:	Kanamycin resistance
XbaI:	Cleavage site of the restriction enzyme XbaI

pknD': Incomplete fragment of the pknD gene from
DM1547

What is claimed is:

1. An isolated polynucleotide from corynebacteria which contains a polynucleotide sequence coding for the pknD gene and is selected from the group comprising:
 - 5 a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2,
 - b) a polynucleotide coding for a polypeptide containing an amino acid sequence which is at least 70%
10 identical to the amino acid sequence of SEQ ID No. 2,
 - c) a polynucleotide which is complementary to the polynucleotides of a) or b), and
 - 15 d) a polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c),the polypeptide preferably having the activity of protein kinase D.
- 20 2. A polynucleotide as claimed in claim 1 which is a preferably recombinant DNA replicable in corynebacteria.
3. A polynucleotide as claimed in claim 1 which is an RNA.
4. A polynucleotide as claimed in claim 2 which contains the nucleic acid sequence as shown in SEQ ID No. 1.
- 25 5. A replicatable DNA as claimed in claim 2 which contains:
 - (i) the nucleotide sequence shown in SEQ ID No. 1,
or

(ii) at least one sequence corresponding to sequence (i) within the degeneracy of the genetic code, or

5 (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) neutral sense mutations in (i).

10 6. A replicable DNA as claimed in claim 5 wherein the hybridization is carried out under a stringency corresponding to at most 2x SSC.

7. A polynucleotide sequence as claimed in claim 1 which codes for a polypeptide containing the amino acid sequence shown in SEQ ID No. 2.

15 8. Corynebacteria in which the *pknD* gene is amplified and, in particular, overexpressed.

9. A fermentation process for the preparation of L-amino acids, especially L-lysine, wherein the following steps are carried out:

20 a) fermentation of the corynebacteria producing the desired L-amino acid, in which at least the endogenous *pknD* gene or nucleotide sequences coding therefor are amplified and, in particular, overexpressed,

25 b) enrichment of the L-amino acid in the medium or in the cells of the bacteria, and

c) isolation of the L-amino acid.

10. The process as claimed in claim 9 wherein bacteria are used in which other genes of the biosynthetic pathway of the desired L-amino acid are additionally amplified.

11. The process as claimed in claim 9 wherein bacteria are used in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partially switched off.
- 5 12. The process as claimed in claim 9 wherein a strain transformed with a plasmid vector is used and the plasmid vector carries the nucleotide sequence coding for the *pknD* gene.
- 10 13. The process as claimed in claim 9 wherein the expression of the polynucleotide(s) coding for the *pknD* gene is amplified and, in particular, overexpressed.
14. The process as claimed in claim 9 wherein the catalytic properties of the polypeptide (enzyme protein) for which the *pknD* polynucleotide codes are enhanced.
- 15 15. The process as claimed in claim 9 wherein, for the production of L-amino acids, coryneform microorganisms are fermented in which one or more endogenous genes selected from the following group are simultaneously amplified and, in particular, overexpressed:
- 20 15.1 the *dapA* gene coding for dihydrodipicolinate synthase,
- 15.2 the *gap* gene coding for glyceraldehyde 3-phosphate dehydrogenase,
- 25 15.3 the *tpi* gene coding for triose phosphate isomerase,
- 15.4 the *pgk* gene coding for 3-phosphoglycerate kinase,
- 15.5 the *zwf* gene coding for glucose-6-phosphate dehydrogenase,
- 30 15.6 the *pyc* gene coding for pyruvate carboxylase,

- 15.7 the lysC gene coding for a feedback-resistant aspartate kinase,
- 15.8 the lysE gene coding for lysine export,
- 5 15.9 the hom gene coding for homoserine dehydrogenase,
- 15.10 the ilvA gene coding for threonine dehydratase or the ilvA(Fbr) allele coding for a feedback-resistant threonine dehydratase,
- 10 15.11 the ilvBN gene coding for acetohydroxy acid synthase,
- 15.12 the ilvD gene coding for dihydroxy acid dehydratase, or
- 15.13 the zwal gene coding for the Zwal protein.
- 15 16. The process as claimed in claim 9 wherein, for the production of L-amino acids, coryneform microorganisms are fermented in which one or more genes selected from the following group are simultaneously attenuated:
- 16.1 the pck gene coding for phosphoenol pyruvate carboxykinase,
- 20 16.2 the pgi gene coding for glucose-6-phosphate isomerase,
- 16.3 the poxB gene coding for pyruvate oxidase, or
- 16.4 the zwa2 gene coding for the Zwa2 protein.
- 25 17. Escherichia coli strain S17-1/pK18mobsacB_pknD_XL as DSM 14410 deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Brunswick, Germany.

18. Corynebacteria which contain a vector carrying a polynucleotide as claimed in claim 1.

19. The process as claimed in one or more of claims 9-16, wherein microorganisms of the species *Corynebacterium glutamicum* are used.

20. The process as claimed in claim 19, wherein the *Corynebacterium* strain S17-1/pK18mobsacB_pknD_XL is used.

21. A method of detecting RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes, which code for protein kinase D or have a high degree of similarity to the sequence of the pknD gene, wherein the polynucleotide containing the polynucleotide sequences as claimed in claims 1, 2, 3 or 4 is used as hybridization probes.

22. The method as claimed in claim 21 wherein arrays, micro-arrays or DNA chips are used.

23. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences between positions 661 and 669 in SEQ ID No. 2 are modified by amino acid exchange.

24. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain any other proteogenic amino acid except glutamic acid in position 664 in SEQ ID No. 2.

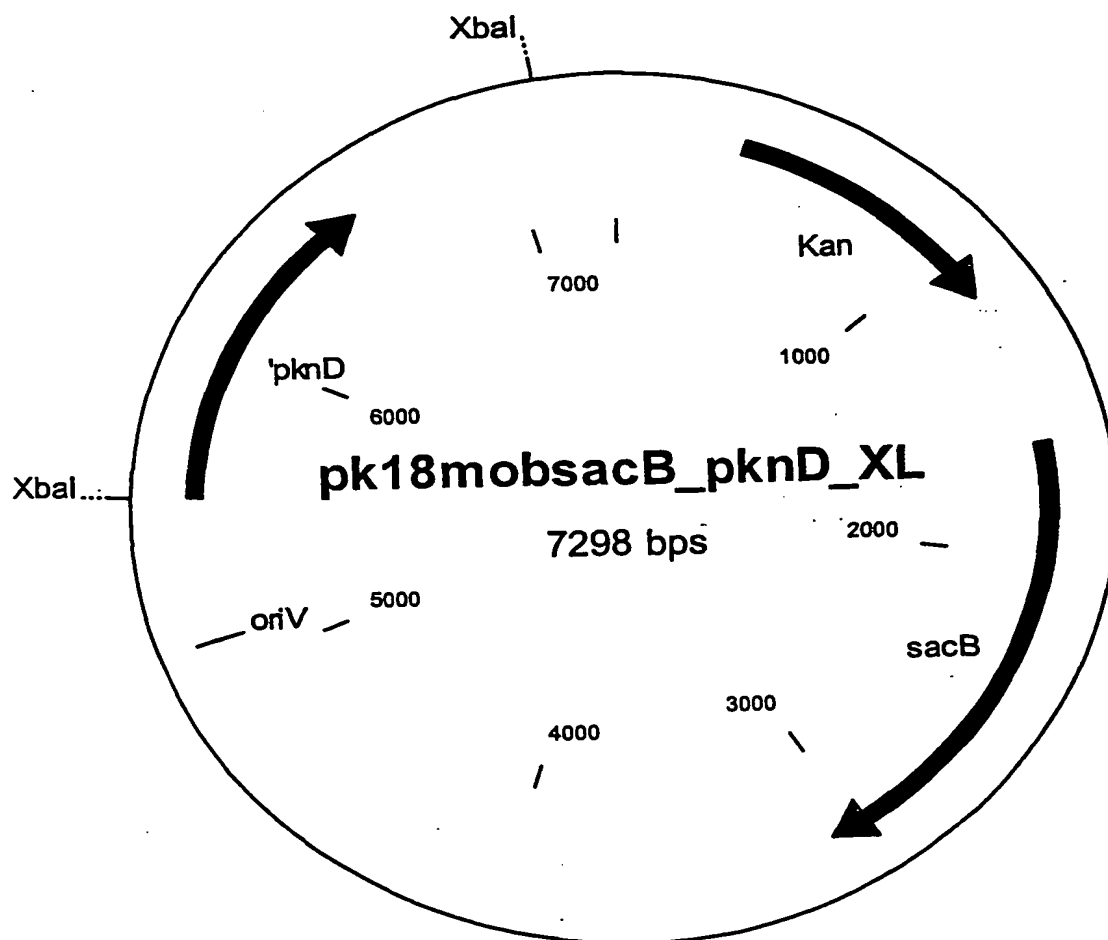
25. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain L-lysine or L-arginine in position 664 in SEQ ID No. 2.

26. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid

sequence contains L-lysine in position 664 in SEQ ID No. 2.

27. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain any other proteogenic amino acid except glycine in position 666 in SEQ ID No. 2.
28. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain L-serine or L-threonine in position 666 in SEQ ID No. 2.
29. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequence contains L-serine in position 666 in SEQ ID No. 2.
30. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequence contains glycine in position 664 and L-serine in position 666, shown in SEQ ID No. 4.
31. A DNA as claimed in claim 30 wherein said DNA contains the nucleobase adenine in position 2501 and the nucleobase adenine in position 2507, shown in SEQ ID No. 3.
32. Corynebacteria which contain a DNA as claimed in one or more of claims 23 to 31.
33. *Corynebacterium glutamicum* DM1547 deposited as DSM13994 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Brunswick, Germany.

Figure 1: Map of the plasmid pk18mobsacB pknD XL



<110> Degussa AG

<120> Nucleotide sequences coding for the pknD gene

<130> 000507 BT

<140>

<141>

<160> 6

<170> PatentIn Ver. 2.1

<210> 1

<211> 3341

<212> DNA

<212> DNA
<213> *Corynebacterium glutamicum*

. **<220>**

<221> CDS

<222> (512) .. (2731)

<223> pknD gene

<400> 1

<400> 1
agaacgccat tgcttgagcg cgtcgcataa cttcacgagc caactggcca tgaagtgcac 60

agaacgcctat cgcttcgagcg cgcttcgagcg
cgatggggcg accaggaagg gtctcgtctt cggtaaacag gaacgcgagg atttcctcgt 120

cgatggggcg accaggaagc gacgggacac 170
cgctgaagcc accgtcggca agcaaggcaa taactccagg gatgaaacgc ttggtgtttt 180

ccttcttgggt gctcaggaaa gcttctggaa tgtagcgaat accgtcgcgc cggaccacga 240

tcaatttgtg ttcatgacc agatccatca ccttggtgac aacaacgccg aggcgctcgg 300

ctgtctccgg aagggtcagc aatggttcat tgtcgggcag ggcgaaggaa gattcattgt 360

tggaactcac agtcttaatt tagctggttc gagctctaatt ggagaatctt taggggtattt 420

ctgcgcgtgc cgggaatgaa agcaccttct tgacctttga aaacaggatg tcaactaccac 480

ctgcgcgtgc cgggaatgaa tga
 tttttgtgta ccttccgaca tactggaacg c atg gca aac ttg aag gtc ggt 532
 Met Ala Asn Leu Lys Val Gly

Met Ala Asn Leu Lys Val Gly
1 5 500

gac gtt tta gag gac agg tat cgg att gaa act ccg att gcc cgg ggt 580
 Asp Val Leu Glu Arg Tyr Arg Ile Glu Thr Pro Ile Ala Arg Gly

gac gcc tta gag ggc
Asp Val Leu Glu Asp Arg Tyr Arg Ile Glu Thr Pro Ile Ala Arg Gly
10 15 20

ggt atg tct acc gtg tac agg tgc ctt gat ctt cgt tta gga cgt tcc 628
 ggt atg tct acc gtg tac agg tgc ctt gat ctt cgt tta gga cgt tcc

Gly Met 25 Thr Val Tyr Arg Cys Leu Asp Leu Arg 35 Leu Gly Arg Ser

atg gcg ctt aaa gtc atg gaa gaa gat ttc gtt gat gat ccc att ttc 676
Met Met Glu Glu Asp Phe Val Asp Asp Pro Ile Phe

atg gcg ctc acc ggc tgg
Met Ala Leu Lys Val Met Glu Glu Asp Phe Val Asp Asp Pro Ile Thr
40 45 50 55

WO 02/22632

cgg cag cgt ttc cgt agg gaa gct cgg tca atg gcg cag cta aat cat Arg Gln Arg Phe Arg Arg Glu Ala Arg Ser Met Ala Gln Leu Asn His 60 65 70	724
cca aat ttg gtc aat gtg tat gat ttt tcc gct act gac ggt ttg gtg Pro Asn Leu Val Asn Val Tyr Asp Phe Ser Ala Thr Asp Gly Leu Val 75 80 85	772
tat ctg gtg atg gag tta atc act ggt ggc acc ttg cgt gag ttg ctg Tyr Leu Val Met Glu Leu Ile Thr Gly Gly Thr Leu Arg Glu Leu Leu 90 95 100	820
gct gag cgg gga cct atg ccc ccg cat gct gct gtg ggc gtt atg cgt Ala Glu Arg Gly Pro Met Pro Pro His Ala Ala Val Gly Val Met Arg 105 110 115	868
ggg gtg ctc acg ggt ctc gcg gct gcc cac cgg gcg ggc atg gtg cac Gly Val Leu Thr Gly Leu Ala Ala Ala His Arg Ala Gly Met Val His 120 125 130 135	916
cgg gat atc aag cct gac aac gtg ttg atc aat agt gat cac cag gtg Arg Asp Ile Lys Pro Asp Asn Val Leu Ile Asn Ser Asp His Gln Val 140 145 150	964
aaa ctg tct gat ttc ggc ttg gtt cga gcg gct cac gcc ggc cag tct Lys Leu Ser Asp Phe Gly Leu Val Arg Ala Ala His Ala Gly Gln Ser 155 160 165	1012
cag gac aat cag att gtg ggc acg gtg gct tat ctt tcc cct gag cag Gln Asp Asn Gln Ile Val Gly Thr Val Ala Tyr Leu Ser Pro Glu Gln 170 175 180	1060
gtt gag ggc ggt gag atc ggg ccg gcc agc gac gtg tat tcg gca ggc Val Glu Gly Gly Glu Ile Gly Pro Ala Ser Asp Val Tyr Ser Ala Gly 185 190 195	1108
att gtg ctc ttt gag ctg ctc aca ggc acc acg cct ttt tcg ggc gag Ile Val Leu Phe Glu Leu Leu Thr Gly Thr Thr Pro Phe Ser Gly Glu 200 205 210 215	1156
gat gat ctc gac cat gca tac gcc cgc ctt acg gaa gtc gtg ccg gca Asp Asp Leu Asp His Ala Tyr Ala Arg Leu Thr Glu Val Val Pro Ala 220 225 230	1204
ccg agt tcg ctt atc gac ggc gtc ccc tcc ctc atc gat gag ctt gtc Pro Ser Ser Leu Ile Asp Gly Val Pro Ser Leu Ile Asp Glu Leu Val 235 240 245	1252
gcg aca gct acc tcc att aat cct gag gat cgt ttc gat gat tct gga Ala Thr Ala Thr Ser Ile Asn Pro Glu Asp Arg Phe Asp Asp Ser Gly 250 255 260	1300
gag ttt ttg tcc gca ctg gaa gat gtc gca aca gag ttg agc ttg ccg Glu Phe Leu Ser Ala Leu Glu Asp Val Ala Thr Glu Leu Ser Leu Pro 265 270 275	1348
gct ttc cgg gtc cct gtg ccg gtt aat tcc gca gcc aat agg gct aat Ala Phe Arg Val Pro Val Pro Val Asn Ser Ala Ala Asn Arg Ala Asn 280 285 290 295	1396

WO 02/22632

gcc cag gtc ccg gat gct cag cca act gat atg ttt acc acc cat atc 1444
 Ala Gln Val Pro Asp Ala Gln Pro Thr Asp Met Phe Thr Thr His Ile
 300 305 310

ccc aag act cct gag cct gat cac act gcg atc att ccg gtg gcc tca 1492
 Pro Lys Thr Pro Glu Pro Asp His Thr Ala Ile Ile Pro Val Ala Ser
 315 320 325

gca aat gag acg tcg att ctg cct gcg caa aac atg gca caa aat atg 1540
 Ala Asn Glu Thr Ser Ile Leu Pro Ala Gln Asn Met Ala Gln Asn Met
 330 335 340

gcg cag aat ccg ctg caa cct ccg gaa cct gat ttc gcc ccg gag cca 1588
 Ala Gln Asn Pro Leu Gln Pro Pro Glu Pro Asp Phe Ala Pro Glu Pro
 345 350 355

cct ccg gac aca gcg ctg aat att caa gat caa gag ctt gcg cgc gcc 1636
 Pro Pro Asp Thr Ala Leu Asn Ile Gln Asp Gln Glu Leu Ala Arg Ala
 360 365 370 375

gat gag cca gaa att aat acc gtc agc aat cgt tcc aaa ttg aag ctg 1684
 Asp Glu Pro Glu Ile Asn Thr Val Ser Asn Arg Ser Lys Leu Lys Leu
 380 385 390

acg ttg tgg tca att ttc gtg gtc gca gtg atc gct gct gtt gct gtt 1732
 Thr Leu Trp Ser Ile Phe Val Val Ala Val Ile Ala Ala Val Ala Val
 395 400 405

ggc ggt tgg tgg ttc ggt tca ggc cgt tac ggt gag att ccg cag gtg 1780
 Gly Gly Trp Trp Phe Gly Ser Gly Arg Tyr Gly Glu Ile Pro Gln Val
 410 415 420

ttg ggc atg gat gag gtc cag gca gta gct gtt gta gag gaa gct ggt 1828
 Leu Gly Met Asp Glu Val Gln Ala Val Ala Val Val Glu Glu Ala Gly
 425 430 435

ttc gtg gca gtg gct gaa cct cag tat gac aat gag gtt ccc act ggt 1876
 Phe Val Ala Val Ala Glu Pro Gln Tyr Asp Asn Glu Val Pro Thr Gly
 440 445 450 455

tcg att att ggg act gaa cct tct ttt ggt gag cgc ctt cct cgc ggc 1924
 Ser Ile Ile Gly Thr Glu Pro Ser Phe Gly Glu Arg Leu Pro Arg Gly
 460 465 470

gag gat gtt tct gtc ctc gtc tct caa ggg cgt ccc gtg gtg ccg gat 1972
 Glu Asp Val Ser Val Leu Val Ser Gln Gly Arg Pro Val Val Pro Asp
 475 480 485

ctt agc gag gat cga tcc tta agc acc gtt cgt gaa gag ttg gaa cag 2020
 Leu Ser Glu Asp Arg Ser Leu Ser Thr Val Arg Glu Glu Leu Glu Gln
 490 495 500

cgc acg ttc gtc tgg gtt gat ggc cca ggt gaa tat tct gac gat gtt 2068
 Arg Thr Phe Val Trp Val Asp Gly Pro Gly Glu Tyr Ser Asp Asp Val
 505 510 515

cca gaa gga caa gta gtt tct ttt aca ccg tcg tca ggc acg cag ctt 2116
 Pro Glu Gly Gln Val Val Ser Phe Thr Pro Ser Ser Gly Thr Gln Leu
 520 525 530 535

WO 02/22632

gat gtt ggt gaa acc gtg cag atc cat ttg agc cga ggc ccc gcc ccg 2164
 Asp Val Gly Glu Thr Val Gln Ile His Leu Ser Arg Gly Pro Ala Pro 550
 540

gtt gag att cct gat gtc tct ggc atg gga gtg gat cag gca aca cgt 2212
 Val Glu Ile Pro Asp Val Ser Gly Met Gly Val Asp Gln Ala Thr Arg 565
 555

gtg ttg gag cgc gca ggt ttg agc gtc gag cgt act gaa gaa ggc ttt 2260
 Val Leu Glu Arg Ala Gly Leu Ser Val Glu Arg Thr Glu Glu Gly Phe 580
 570

gat gct gag aca cca aat ggt gat gtc tac ggg act tcg ccc aag gta 2308
 Asp Ala Glu Thr Pro Asn Gly Asp Val Tyr Gly Thr Ser Pro Lys Val 595
 585

tct act gag gtc aag cgc gga acc tct gtt gtg ctg cag gtg tcc aat 2356
 Ser Thr Glu Val Lys Arg Gly Thr Ser Val Val Leu Gln Val Ser Asn 615
 600

gct att tcg gta ccg gat gtg gtg ggt atg acc aag gac gaa gcc acc 2404
 Ala Ile Ser Val Pro Asp Val Val Gly Met Thr Lys Asp Glu Ala Thr 630
 620

gcg gcg ctt gcg gaa gaa gga ttg gtc gtg gcg tcg aca agc att att 2452
 Ala Ala Leu Ala Glu Glu Gly Leu Val Val Ala Ser Thr Ser Ile Ile 645
 635

cct ggt gag gcg gcg agc tcc gct gac gcc gtc gtg acc gtc gag cct 2500
 Pro Gly Glu Ala Ala Ser Ser Ala Asp Ala Val Val Thr Val Glu Pro 660
 650

gaa tcc ggc agc cgc gtt gat cca gcg cat ccg cag gtc agc ctc ggg 2548
 Glu Ser Gly Ser Arg Val Asp Pro Ala His Pro Gln Val Ser Leu Gly 675
 665

tta gct ggg gag att caa gtt cca agc gtg gtt gga cgt aag gtt agc 2596
 Leu Ala Gly Glu Ile Gln Val Pro Ser Val Val Gly Arg Lys Val Ser 695
 680

gat gct cga agc att ctg gaa gaa gcc ggt tta acg ctg aca act gat 2644
 Asp Ala Arg Ser Ile Leu Glu Glu Ala Gly Leu Thr Leu Thr Thr Asp 710
 700

gcg gac gac aac gat cga att tat agt caa acc cct cgt gca cgc agc 2692
 Ala Asp Asp Asn Asp Arg Ile Tyr Ser Gln Thr Pro Arg Ala Arg Ser 725
 715

gaa gtc tcg gta ggg gga gaa gtt aca gta agg gcg ttt tagtggttcc 2741
 Glu Val Ser Val Gly Gly Glu Val Thr Val Arg Ala Phe 740
 730

ctcggttgacg caatggcgaa aacctgctct catcctggcc attttgacgg tgctaggcgt 2801
 actcctgacc cattgggttcg cctggccact cacctggccg ctggggctgc gtcttcccgt 2861
 tgatgtagag gtgtactggc aggggtgcgcg cgagttttgg ctgcgcgatg atctctacga 2921
 catcaggtat gacaccactt tcgacaactt gccgttcacc tatccccctt tcggtgcgtt 2981
 ggtgttcacc ccattgtggt ggattcatga cctctttggt cttctcgtca ccgaacgtgt 3041
 cttcgcgcta atcacgctgc tcaccaccta cgctgtggca gttttcctgc tccgcctggc 3101

cggcgtgcgc gatcgtgtgt gggaattcgt cgcattcgca gccctgctcg tgtccgcgcc 3161
 ggtgtatttc aactcaata ttgggcaaat aaacgtcatg ctcatggctt taacgctttt 3221
 cgacgtcgcc ctccccgca gcacgcgcca ttcaggcgtg ctcaaatacg tgccactcgg 3281
 cgtactcacc ggcattgccg ctgcgatcaa actaacccca ctagtggttcg ggctgtattt 3341

<210> 2

<211> 740

<212> PRT

<213> Corynebacterium glutamicum

<400> 2

Met Ala Asn Leu Lys Val Gly Asp Val Leu Glu Asp Arg Tyr Arg Ile
 1 5 10 15
 Glu Thr Pro Ile Ala Arg Gly Gly Met Ser Thr Val Tyr Arg Cys Leu
 20 25 30
 Asp Leu Arg Leu Gly Arg Ser Met Ala Leu Lys Val Met Glu Glu Asp
 35 40 45
 Phe Val Asp Asp Pro Ile Phe Arg Gln Arg Phe Arg Arg Glu Ala Arg
 50 55 60
 Ser Met Ala Gln Leu Asn His Pro Asn Leu Val Asn Val Tyr Asp Phe
 65 70 75 80
 Ser Ala Thr Asp Gly Leu Val Tyr Leu Val Met Glu Leu Ile Thr Gly
 85 90 95
 Gly Thr Leu Arg Glu Leu Leu Ala Glu Arg Gly Pro Met Pro Pro His
 100 105 110
 Ala Ala Val Gly Val Met Arg Gly Val Leu Thr Gly Leu Ala Ala Ala
 115 120 125
 His Arg Ala Gly Met Val His Arg Asp Ile Lys Pro Asp Asn Val Leu
 130 135 140
 Ile Asn Ser Asp His Gln Val Lys Leu Ser Asp Phe Gly Leu Val Arg
 145 150 155 160
 Ala Ala His Ala Gly Gln Ser Gln Asp Asn Gln Ile Val Gly Thr Val
 165 170 175
 Ala Tyr Leu Ser Pro Glu Gln Val Glu Gly Gly Glu Ile Gly Pro Ala
 180 185 190
 Ser Asp Val Tyr Ser Ala Gly Ile Val Leu Phe Glu Leu Leu Thr Gly
 195 200 205
 Thr Thr Pro Phe Ser Gly Glu Asp Asp Leu Asp His Ala Tyr Ala Arg
 210 215 220

Leu Thr Glu Val Val Pro Ala Pro Ser Ser Leu Ile Asp Gly Val Pro
 225 230 235 240
 Ser Leu Ile Asp Glu Leu Val Ala Thr Ala Thr Ser Ile Asn Pro Glu
 245 250 255
 Asp Arg Phe Asp Asp Ser Gly Glu Phe Leu Ser Ala Leu Glu Asp Val
 260 265 270
 Ala Thr Glu Leu Ser Leu Pro Ala Phe Arg Val Pro Val Pro Val Asn
 275 280 285
 Ser Ala Ala Asn Arg Ala Asn Ala Gln Val Pro Asp Ala Gln Pro Thr
 290 295 300
 Asp Met Phe Thr Thr His Ile Pro Lys Thr Pro Glu Pro Asp His Thr
 305 310 315 320
 Ala Ile Ile Pro Val Ala Ser Ala Asn Glu Thr Ser Ile Leu Pro Ala
 325 330 335
 Gln Asn Met Ala Gln Asn Met Ala Gln Asn Pro Leu Gln Pro Pro Glu
 340 345 350
 Pro Asp Phe Ala Pro Glu Pro Pro Pro Asp Thr Ala Leu Asn Ile Gln
 355 360 365
 Asp Gln Glu Leu Ala Arg Ala Asp Glu Pro Glu Ile Asn Thr Val Ser
 370 375 380
 Asn Arg Ser Lys Leu Lys Leu Thr Leu Trp Ser Ile Phe Val Val Ala
 385 390 395 400
 Val Ile Ala Ala Val Ala Val Gly Gly Trp Trp Phe Gly Ser Gly Arg
 405 410 415
 Tyr Gly Glu Ile Pro Gln Val Leu Gly Met Asp Glu Val Gln Ala Val
 420 425 430
 Ala Val Val Glu Glu Ala Gly Phe Val Ala Val Ala Glu Pro Gln Tyr
 435 440 445
 Asp Asn Glu Val Pro Thr Gly Ser Ile Ile Gly Thr Glu Pro Ser Phe
 450 455 460
 Gly Glu Arg Leu Pro Arg Gly Glu Asp Val Ser Val Leu Val Ser Gln
 465 470 475 480
 Gly Arg Pro Val Val Pro Asp Leu Ser Glu Asp Arg Ser Leu Ser Thr
 485 490 495
 Val Arg Glu Glu Leu Glu Gln Arg Thr Phe Val Trp Val Asp Gly Pro
 500 505 510
 Gly Glu Tyr Ser Asp Asp Val Pro Glu Gly Gln Val Val Ser Phe Thr
 515 520 525
 Pro Ser Ser Gly Thr Gln Leu Asp Val Gly Glu Thr Val Gln Ile His
 530 535 540

Leu Ser Arg Gly Pro Ala Pro Val Glu Ile Pro Asp Val Ser Gly Met
 545 550 555 560
 Gly Val Asp Gln Ala Thr Arg Val Leu Glu Arg Ala Gly Leu Ser Val
 565 570 575
 Glu Arg Thr Glu Glu Gly Phe Asp Ala Glu Thr Pro Asn Gly Asp Val
 580 585 590
 Tyr Gly Thr Ser Pro Lys Val Ser Thr Glu Val Lys Arg Gly Thr Ser
 595 600 605
 Val Val Leu Gln Val Ser Asn Ala Ile Ser Val Pro Asp Val Val Gly
 610 615 620
 Met Thr Lys Asp Glu Ala Thr Ala Ala Leu Ala Glu Glu Gly Leu Val
 625 630 635 640
 Val Ala Ser Thr Ser Ile Ile Pro Gly Glu Ala Ala Ser Ser Ala Asp
 645 650 655
 Ala Val Val Thr Val Glu Pro Glu Ser Gly Ser Arg Val Asp Pro Ala
 660 665 670
 His Pro Gln Val Ser Leu Gly Leu Ala Gly Glu Ile Gln Val Pro Ser
 675 680 685
 Val Val Gly Arg Lys Val Ser Asp Ala Arg Ser Ile Leu Glu Glu Ala
 690 695 700
 Gly Leu Thr Leu Thr Thr Asp Ala Asp Asp Asn Asp Arg Ile Tyr Ser
 705 710 715 720
 Gln Thr Pro Arg Ala Arg Ser Glu Val Ser Val Gly Gly Glu Val Thr
 725 730 735
 Val Arg Ala Phe
 740

<210> 3
 <211> 3341
 <212> DNA
 <213> Corynebacterium glutamicum

<220>
 <221> CDS
 <222> (512)..(2731)
 <223> Allele pknD-1547

<220>
 <221> misc feature
 <222> (2501)
 <223> G-A Transition

<220>
 <221> misc feature
 <222> (2507)
 <223> G-A Transition

<400> 3
 agaacgccat tgcttgagcg cgtcgcataa cttcaccgagc caactggcca tgaagtgcac 60

8

WO 02/22632

gtt gag ggc ggt gag atc ggg ccg gcc agc gac Val Glu Gly Gly Glu Ile Gly Pro Ala Ser Asp 185 190 195	gtg tat tcg gca ggc Val Tyr Ser Ala Gly	1108
att gtg ctc ttt gag ctg ctc aca ggc acc acg cct ttt tcg ggc gag Ile Val Leu Phe Glu Leu Leu Thr Gly Thr 200 205 210 215		1156
gat gat ctc gac cat gca tac gcc cgc ctt acg gaa gtc gtg ccg gca Asp Asp Leu Asp His Ala Tyr Ala Arg Leu Thr Glu Val Val Pro Ala 220 225 230		1204
ccg agt tcg ctt atc gac ggc gtc ccc tcc ctc atc gat gag ctt gtc Pro Ser Ser Leu Ile Asp Gly Val Pro Ser Leu Ile Asp Glu Leu Val 235 240 245		1252
gcg aca gct acc tcc att aat cct gag gat cgt ttc gat gat tct gga Ala Thr Ala Thr Ser Ile Asn Pro Glu Asp Arg Phe Asp Asp Ser Gly 250 255 260		1300
gag ttt ttg tcc gca ctg gaa gat gtc gca aca gag ttg agc ttg ccg Glu Phe Leu Ser Ala Leu Glu Asp Val Ala Thr Glu Leu Ser Leu Pro 265 270 275		1348
gct ttc cgg gtc cct gtg ccg gtt aat tcc gca gcc aat agg gct aat Ala Phe Arg Val Pro Val Pro Val Asn Ser Ala Ala Asn Arg Ala Asn 280 285 290 295		1396
gcc cag gtc ccg gat gct cag cca act gat atg ttt acc acc cat atc Ala Gln Val Pro Asp Ala Gln Pro Thr Asp Met Phe Thr Thr His Ile 300 305 310		1444
ccc aag act cct gag cct gat cac act gcg atc att ccg gtg gcc tca Pro Lys Thr Pro Glu Pro Asp His Thr Ala Ile Ile Pro Val Ala Ser 315 320 325		1492
gca aat gag acg tcg att ctg cct gcg caa aac atg gca caa aat atg Ala Asn Glu Thr Ser Ile Leu Pro Ala Gln Asn Met Ala Gln Asn Met 330 335 340		1540
gcg cag aat ccg ctg caa cct ccg gaa cct gat ttc gcc ccg gag cca Ala Gln Asn Pro Leu Gln Pro Pro Glu Pro Asp Phe Ala Pro Glu Pro 345 350 355		1588
cct ccg gac aca gcg ctg aat att caa gat caa gag ctt gcg cgc gcc Pro Pro Asp Thr Ala Leu Asn Ile Gln Asp Gln Glu Leu Ala Arg Ala 360 365 370 375		1636
gat gag cca gaa att aat acc gtc agc aat cgt tcc aaa ttg aag ctg Asp Glu Pro Glu Ile Asn Thr Val Ser Asn Arg Ser Lys Leu Lys Leu 380 385 390		1684
acg ttg tgg tca att ttc gtg gtc gca gtg atc gct gct gtt gct gtt Thr Leu Trp Ser Ile Phe Val Val Ala Val Ile Ala Ala Val Ala Val 395 400 405		1732

WO 02/22632

ggc ggt tgg tgg ttc ggt tca ggc cgt tac ggt gag att ccg cag gtg Gly Gly Trp Trp Phe Gly Ser Gly Arg Tyr Gly Glu Ile Pro Gln Val 410 415 420	1780
ttg ggc atg gat gag gtc cag gca gta gct gtt gta gag gaa gct ggt Leu Gly Met Asp Glu Val Gln Ala Val Ala Val Glu Glu Ala Gly 425 430 435	1828
ttc gtg gca gtg gct gaa cct cag tat gac aat gag gtt ccc act ggt Phe Val Ala Val Ala Glu Pro Gln Tyr Asp Asn Glu Val Pro Thr Gly 440 445 450 455	1876
tcg att att ggg act gaa cct tct ttt ggt gag cgc ctt cct cgc ggc Ser Ile Ile Gly Thr Glu Pro Ser Phe Gly Glu Arg Leu Pro Arg Gly 460 465 470	1924
gag gat gtt tct gtc ctc gtc tct caa ggg cgt ccc gtg gtg ccg gat Glu Asp Val Ser Val Leu Val Ser Gln Gly Arg Pro Val Val Pro Asp 475 480 485	1972
ctt agc gag gat cga tcc tta agc acc gtt cgt gaa gag ttg gaa cag Leu Ser Glu Asp Arg Ser Leu Ser Thr Val Arg Glu Glu Leu Glu Gln 490 495 500	2020
cgc acg ttc gtc tgg gtt gat ggc cca ggt gaa tat tct gac gat gtt Arg Thr Phe Val Trp Val Asp Gly Pro Gly Glu Tyr Ser Asp Asp Val 505 510 515	2068
cca gaa gga caa gta gtt tct ttt aca ccg tcg tca ggc acg cag ctt Pro Glu Gly Gln Val Val Ser Phe Thr Pro Ser Ser Gly Thr Gln Leu 520 525 530 535	2116
gat gtt ggt gaa acc gtg cag atc cat ttg agc cga ggc ccc gcc ccg Asp Val Gly Glu Thr Val Gln Ile His Leu Ser Arg Gly Pro Ala Pro 540 545 550	2164
gtt gag att cct gat gtc tct ggc atg gga gtg gat cag gca aca cgt Val Glu Ile Pro Asp Val Ser Gly Met Gly Val Asp Gln Ala Thr Arg 555 560 565	2212
gtg ttg gag cgc gca ggt ttg agc gtc gag cgt act gaa gaa ggc ttt Val Leu Glu Arg Ala Gly Leu Ser Val Glu Arg Thr Glu Glu Gly Phe 570 575 580	2260
gat gct gag aca cca aat ggt gat gtc tac ggg act tcg ccc aag gta Asp Ala Glu Thr Pro Asn Gly Asp Val Tyr Gly Thr Ser Pro Lys Val 585 590 595	2308
tct act gag gtc aag cgc gga acc tct gtt gtg ctg cag gtg tcc aat Ser Thr Glu Val Lys Arg Gly Thr Ser Val Val Leu Gln Val Ser Asn 600 605 610 615	2356
gct att tcg gta ccg gat gtg gtg ggt atg acc aag gac gaa gcc acc Ala Ile Ser Val Pro Asp Val Val Gly Met Thr Lys Asp Glu Ala Thr 620 625 630	2404
gcg gcg ctt gcg gaa gaa gga ttg gtc gtg gcg tcg aca agc att att Ala Ala Leu Ala Glu Glu Gly Leu Val Val Ala Ser Thr Ser Ile Ile 635 640 645	2452

WO 02/22632

cct ggt gag gcg gcg agc tcc gct gac gcc gtc gtg acc gtc gag cct 2500
 Pro Gly Glu Ala Ala Ser Ser Ala Asp Ala Val Val Thr Val Glu Pro 660
 650 655

aaa tcc agc agc cgc gtt gat cca gcg cat ccg cag gtc agc ctc ggg 2548
 Lys Ser Ser Ser Arg Val Asp Pro Ala His Pro Gln Val Ser Leu Gly 675
 665 670

tta gct ggg gag att caa gtt cca agc gtg gtt gga cgt aag gtt agc 2596
 Leu Ala Gly Glu Ile Gln Val Pro Ser Val Val Gly Arg Lys Val Ser 695
 680 685 690

gat gct cga agc att ctg gaa gaa gcc ggt tta acg ctg aca act gat 2644
 Asp Ala Arg Ser Ile Leu Glu Glu Ala Gly Leu Thr Leu Thr Thr Asp 710
 700 705

gcg gac gac aac gat cga att tat agt caa acc cct cgt gca cgc agc 2692
 Ala Asp Asp Asn Asp Arg Ile Tyr Ser Gln Thr Pro Arg Ala Arg Ser 725
 715 720

gaa gtc tcg gta ggg gga gaa gtt aca gta agg gcg ttt tagtggttcc 2741
 Glu Val Ser Val Gly Gly Glu Val Thr Val Arg Ala Phe 740
 730 735

ctggttgacg caatggcgaa aacctgctct catcctggcc attttgacgg tgctaggcgt 2801
 actcctgacc cattggttcg cctggccact cacctggccg ctggggctgc gtcttcccgt 2861
 tgatgtagag gtgtactggc aggggtgcgcg cgagttttgg ctgcccgatg atctctacga 2921
 catcaggtat gacaccaatt tcgacaactt gccgttcacc tatccccctt tcgggtgcgtt 2981
 ggtgttcacc ccattgtggt ggattcatga cctcttttgt cttctcgtca ccgaacgtgt 3041
 cttegcgcta atcacgctgc tcaccaccta cgctgtggca gttttcctgc tccgcctggc 3101
 cggcgtgcgc gatcgtgtgt ggggaattcgt cgcattcgca gccctgctcg tgcgcgcgcc 3161
 ggtgtatttc aactcaata ttgggcaa ataacgtcatg ctcatggctt taacgctttt 3221
 cgacgtcgcc ctcccccgca gcacgcgcca ttcaggcgtg ctcaaatacg tgccactcgg 3281
 cgtactcacc ggcattgccg ctgcgatcaa actaacccca ctagtgttcg ggctgtattt 3341

<210> 4

<211> 740

<212> PRT

<213> Corynebacterium glutamicum

<400> 4

Met Ala Asn Leu Lys Val Gly Asp Val Leu Glu Asp Arg Tyr Arg Ile
 1 5 10 15

Glu Thr Pro Ile Ala Arg Gly Gly Met Ser Thr Val Tyr Arg Cys Leu
 20 25 30

Asp Leu Arg Leu Gly Arg Ser Met Ala Leu Lys Val Met Glu Glu Asp
 35 40 45

WO 02/22632

Phe Val Asp Asp Pro Ile Phe Arg Gln Arg Phe Arg Arg Glu Ala Arg
 50 55 60
 Ser Met Ala Gln Leu Asn His Pro Asn Leu Val Asn Val Tyr Asp Phe
 65 70 75 80
 Ser Ala Thr Asp Gly Leu Val Tyr Leu Val Met Glu Leu Ile Thr Gly
 85 90 95
 Gly Thr Leu Arg Glu Leu Leu Ala Glu Arg Gly Pro Met Pro Pro His
 100 105 110
 Ala Ala Val Gly Val Met Arg Gly Val Leu Thr Gly Leu Ala Ala Ala
 115 120 125
 His Arg Ala Gly Met Val His Arg Asp Ile Lys Pro Asp Asn Val Leu
 130 135 140
 Ile Asn Ser Asp His Gln Val Lys Leu Ser Asp Phe Gly Leu Val Arg
 145 150 155 160
 Ala Ala His Ala Gly Gln Ser Gln Asp Asn Gln Ile Val Gly Thr Val
 165 170 175
 Ala Tyr Leu Ser Pro Glu Gln Val Glu Gly Gly Glu Ile Gly Pro Ala
 180 185 190
 Ser Asp Val Tyr Ser Ala Gly Ile Val Leu Phe Glu Leu Leu Thr Gly
 195 200 205
 Thr Thr Pro Phe Ser Gly Glu Asp Asp Leu Asp His Ala Tyr Ala Arg
 210 215 220
 Leu Thr Glu Val Val Pro Ala Pro Ser Ser Leu Ile Asp Gly Val Pro
 225 230 235 240
 Ser Leu Ile Asp Glu Leu Val Ala Thr Ala Thr Ser Ile Asn Pro Glu
 245 250 255
 Asp Arg Phe Asp Asp Ser Gly Glu Phe Leu Ser Ala Leu Glu Asp Val
 260 265 270
 Ala Thr Glu Leu Ser Leu Pro Ala Phe Arg Val Pro Val Pro Val Asn
 275 280 285
 Ser Ala Ala Asn Arg Ala Asn Ala Gln Val Pro Asp Ala Gln Pro Thr
 290 295 300
 Asp Met Phe Thr Thr His Ile Pro Lys Thr Pro Glu Pro Asp His Thr
 305 310 315 320
 Ala Ile Ile Pro Val Ala Ser Ala Asn Glu Thr Ser Ile Leu Pro Ala
 325 330 335
 Gln Asn Met Ala Gln Asn Met Ala Gln Asn Pro Leu Gln Pro Pro Glu
 340 345 350
 Pro Asp Phe Ala Pro Glu Pro Pro Pro Asp Thr Ala Leu Asn Ile Gln
 355 360 365

Asp Gln Glu Leu Ala Arg Ala Asp Glu Pro Glu Ile Asn Thr Val Ser
 370 375 380
 Asn Arg Ser Lys Leu Lys Leu Thr Leu Trp Ser Ile Phe Val Val Ala
 385 390 395 400
 Val Ile Ala Ala Val Ala Val Gly Gly Trp Trp Phe Gly Ser Gly Arg
 405 410 415
 Tyr Gly Glu Ile Pro Gln Val Leu Gly Met Asp Glu Val Gln Ala Val
 420 425 430
 Ala Val Val Glu Glu Ala Gly Phe Val Ala Val Ala Glu Pro Gln Tyr
 435 440 445
 Asp Asn Glu Val Pro Thr Gly Ser Ile Ile Gly Thr Glu Pro Ser Phe
 450 455 460
 Gly Glu Arg Leu Pro Arg Gly Glu Asp Val Ser Val Leu Val Ser Gln
 465 470 475 480
 Gly Arg Pro Val Val Pro Asp Leu Ser Glu Asp Arg Ser Leu Ser Thr
 485 490 495
 Val Arg Glu Glu Leu Glu Gln Arg Thr Phe Val Trp Val Asp Gly Pro
 500 505 510
 Gly Glu Tyr Ser Asp Asp Val Pro Glu Gly Gln Val Val Ser Phe Thr
 515 520 525
 Pro Ser Ser Gly Thr Gln Leu Asp Val Gly Glu Thr Val Gln Ile His
 530 535 540
 Leu Ser Arg Gly Pro Ala Pro Val Glu Ile Pro Asp Val Ser Gly Met
 545 550 555 560
 Gly Val Asp Gln Ala Thr Arg Val Leu Glu Arg Ala Gly Leu Ser Val
 565 570 575
 Glu Arg Thr Glu Glu Gly Phe Asp Ala Glu Thr Pro Asn Gly Asp Val
 580 585 590
 Tyr Gly Thr Ser Pro Lys Val Ser Thr Glu Val Lys Arg Gly Thr Ser
 595 600 605
 Val Val Leu Gln Val Ser Asn Ala Ile Ser Val Pro Asp Val Val Gly
 610 615 620
 Met Thr Lys Asp Glu Ala Thr Ala Ala Leu Ala Glu Glu Gly Leu Val
 625 630 635 640
 Val Ala Ser Thr Ser Ile Ile Pro Gly Glu Ala Ala Ser Ser Ala Asp
 645 650 655
 Ala Val Val Thr Val Glu Pro Lys Ser Ser Ser Arg Val Asp Pro Ala
 660 665 670
 His Pro Gln Val Ser Leu Gly Leu Ala Gly Glu Ile Gln Val Pro Ser
 675 680 685

Val Val Gly Arg Lys Val Ser Asp Ala Arg Ser Ile Leu Glu Glu Ala
 690 695 700

Gly Leu Thr Leu Thr Thr Asp Ala Asp Asp Asn Asp Arg Ile Tyr Ser
 705 710 715 720

Gln Thr Pro Arg Ala Arg Ser Glu Val Ser Val Gly Gly Glu Val Thr
 725 730 735

Val Arg Ala Phe
 740

<210> 5
 <211> 26
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Description of the artificial sequence: Primer
 pknD_XL-A1

<400> 5
 tctagacggt tgggtggttcg gttcag

26

<210> 6
 <211> 26
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Description of the artificial sequence: Primer
 pknD_XL-E1

<400> 6
 tctagacgcg gcaatgccgg tgagta

26

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/22632 A3

(51) International Patent Classification⁷: C12N 15/31, (72) Inventors: BATHE, Brigitte; Twieten 1, 33154
C12P 13/04, 13/08, C12Q 1/68 // C12R 1:15 Salzotten (DE). SCHRÖDER, Indra; Backsheide
21, 33803 Steinhagen (DE). FARWICK, Mike; Gus-
tav-Adolf-Strasse 11, 33615 Bielefeld (DE). HERMANN,
Thomas; Zirkonstrasse 8, 33739 Bielefeld (DE).

(21) International Application Number: PCT/EP01/10210

(22) International Filing Date:
5 September 2001 (05.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 44 948.4 12 September 2000 (12.09.2000) DE
101 20 094.3 25 April 2001 (25.04.2001) DE

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1,
40474 Düsseldorf (DE).

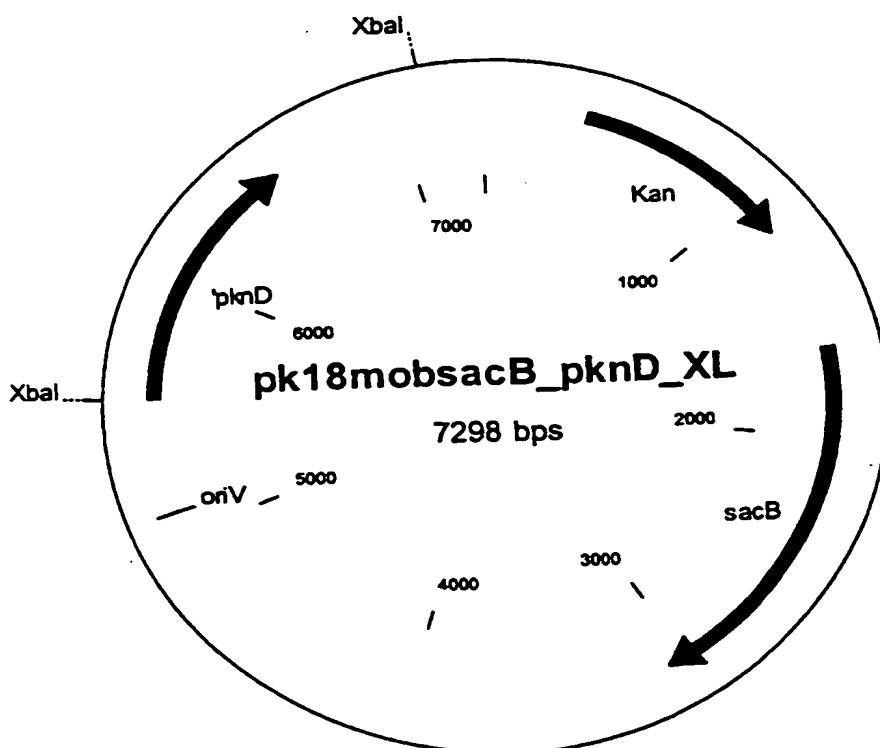
(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA,
ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,

[Continued on next page]

(54) Title: NUCLEOTIDE SEQUENCES CODING FOR THE PKND GENE

Map of the plasmid pk18mobsacB pknD XL



(57) Abstract: The invention relates to an isolated polynucleotide which contains a polynucleotide sequence selected from the group comprising: a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2, b) a polynucleotide coding for a polypeptide containing an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2, c) a polynucleotide which is complementary to the polynucleotides of a) or b), and d) a polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c), and a fermentation process for the preparation of L-amino acids using corynebacteria in which at least the pknD gene is amplified, and to the use, as hybridization probes, of polynucleotides containing the sequences according to the invention.

WO 02/22632 A3



IT, LU, MC, NL, PT, SE, TR). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(88) Date of publication of the international search report:
13 June 2002

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PC1/EP 01/10210

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12P13/04 C12P13/08 C12Q1/68 //C12R1:15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12R C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 411 997 A (SHIMAZAKI KEISHI ET AL) 25 October 1983 (1983-10-25) the whole document	8-11, 13-16
X	WO 00 17379 A (CHEIL JEDANG CORP ; LEE JAE HEUNG (KR); LIM SANG JO (KR); KO JUNG H) 30 March 2000 (2000-03-30) the whole document	8-11, 13-16
X	DATABASE EMBL 'Online! MTV021 Accession Number AL021957, 23 February 1998 (1998-02-23) COLE ET AL. : "Mycobacterium tuberculosis H37Rv complete genome; segment 97/162" XP002191212 accession number AL021957	1-4

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

8 April 2002

Date of mailing of the international search report

22/04/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax (+31-70) 340-3016

Authorized officer

Kools, P

INTERNATIONAL SEARCH REPORT

 Inter. 'onal Application No
 PC1/EP 01/10210

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AV-GAY Y., AND EVERETT M.: "The eukaryotic-like Ser/thr protein kinases of Mycobacterium tuberculosis" TRENDS IN MICROBIOLOGY, vol. 8, no. 5, May 2000 (2000-05), pages 238-244, XP002191211 the whole document	1-33
A	SCHRUMPF B ET AL: "A FUNCTIONALLY SPLIT PATHWAY FOR LYSINE SYNTHESIS IN CORYNEBACTERIUM GLUTAMICUM" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 173, no. 14, July 1991 (1991-07), pages 4510-4516, XP000884180 ISSN: 0021-9193 the whole document	10,11, 15,16
P,X	DATABASE EMBL 'Online! Accession number; AX127150, nt 202141-205620, 10 May 2001 (2001-05-10) NAKAGAWA ET AL.: "Sequence 7066 from Patent EP1108790" XP002191244 accession number AX127150	1-33
P,X	DATABASE EMBL 'Online! Accession number AX125968, 10 May 2001 (2001-05-10) NAKAGAWA ET AL.: "Sequence 5884 from Patent EP1108790" XP002191701 accession number AX125968	1-33
P,X	EP 1 108 790 A (KYOWA HAKKO KOGYO KK) 20 June 2001 (2001-06-20) page 160; table 1	1-33

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 01/10210

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4411997	A	25-10-1983	JP 1426842 C	25-02-1988
			JP 57115186 A	17-07-1982
			JP 62036673 B	07-08-1987
			FR 2497231 A1	02-07-1982
WO 0017379	A	30-03-2000	AU 5763099 A	10-04-2000
			WO 0017379 A2	30-03-2000
EP 1108790	A	20-06-2001	EP 1108790 A2	20-06-2001